Jan M.C. Geuns
editor

Proceedings of the
5th Stevia Symposium 2011
organised by EUSTAS

Stevia: Break-Through in Europe

KULeuven, 28-29 June 2011.

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CHAPTER 12

Determination of Steviol Glycosides in Various Food Categories

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ABSTRACT
The European approval of steviol glycosides (SVGly's) as a food additive is expected in the next few months. It is important to assess the stability of these steviol glycosides after they have been added to different food matrices. We analyzed and tested the stability of SVGly's in semi-skimmed milk, soy drink, fermented milk drink, ice cream, full-fat and skimmed set yoghurt, dry biscuits and jam.

The fat was removed by centrifugation from the dairy and soy drink samples. Proteins were precipitated by addition of acetonitrile and also removed by centrifugation. Samples of jam were extracted with water. Dry biscuits were extracted with ethanol. The resulting samples were concentrated with solid phase extraction and analysed by HPLC on a C_{18} stationary phase and a gradient of acetonitrile / aqueous 25 mM H_3PO_4. The accuracy was checked using a standard addition on

some samples. For assessing the stability of the SVGly's, samples were stored and analyzed periodically.
The results indicate that SVGly's can be analyzed with good precision and accuracy in these food categories. The recovery of SVgly's was between 96 and 99 %. The method was also validated by standard addition, which showed excellent agreement with the external calibration curve. No sign of decomposition of SVGly's was found in any of the samples.

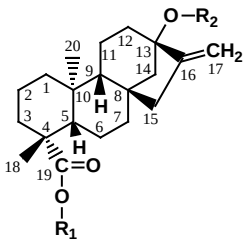
KEYWORDS
Steviol glycosides, analysis, food matrix, stability.

Introduction

As the final EU approval for the addition of steviol glycosides (SVGly) to food is expected very soon, it is important that the analysis and stability of SVGly in various food matrices is thoroughly tested. One has to be certain that these SVGly are sufficiently stable in the matrix, so that the quality and the normal shelf-life of the foodstuff are not compromised by the addition of SVGly.

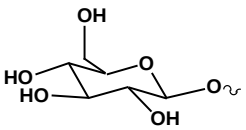
In this study we wish to report on the stability of SVGly which were added to various classes of food. The structures of the SVGly that were analyzed are summarized in Table 1. With the agreement of the companies that contributed to the project, the following food matrices were chosen: semi-skimmed milk, soy drink, fermented milk drink, ice cream, full-fat set yoghurt, skimmed set yoghurt, dry biscuits and jam. Several additions of SVGly were made in the different food matrices and the concentrations were followed as a function of time. This allowed us to assess the stability of the added SVGly during the normal shelf life of the food matrix.

Table 1: Structures of common SVGly (Wood, Allerton, Diehl & Fletcher, 1955)

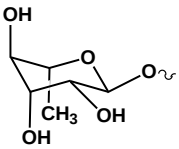


Name and acronym	R_1	R_2	Molecular formula	Molecular mass
Steviol (SV)	H	H	$C_{20}H_{30}O_3$	318.45
Stevioside (Ste)	β-Glc	β-Glc-β-Glc(2→1)	$C_{38}H_{60}O_{18}$	804.87
Steviolbioside (SteB)	H	β-Glc-β-Glc(2→1)	$C_{32}H_{50}O_{13}$	642.73
Rubusoside (Rub)	β–Glc	β-Glc	$C_{32}H_{50}O_{13}$	642.73
Rebaudioside A (RebA)	β-Glc	β-Glc-β-Glc(2→1) | β-Glc(3→1)	$C_{44}H_{70}O_{23}$	967.01
Rebaudioside B (RebB)	H	β-Glc-β-Glc(2→1) | β-Glc(3→1)	$C_{38}H_{60}O_{18}$	804.87
Rebaudioside C (RebC)	β-Glc	β-Glc-α-Rha(2→1) | β-Glc(3→1)	$C_{44}H_{70}O_{22}$	951.01
Rebaudioside F (RebF)	β-Glc	β-Glc-β-Xyl(2→1) | β-Glc(3→1)	$C_{43}H_{68}O_{22}$	936.99
Dulcoside A (DulA)	β-Glc	β-Glc-α-Rha(2→1)	$C_{38}H_{60}O_{17}$	788.87

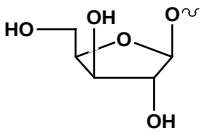
Glc: glucose



Rha: rhamnose



Xyl: xylose



Only a limited number of recent studies about the stability of SVGly in food are published in the recent literature (Clos *et al.*, 2008; Kroyer, 1999; Kroyer, 2010; Prakash *et al.*, 2008; Woelwer-Rieck *et al.*, 2010).

The study by Clos *et al.* (2008) deals with the photostability of RebA and Ste in carbonated soft-drinks. Although it is somewhat outside the scope of the present study, it is however relevant, because Clos studied the stability in rather acidic media. Both the acetal group at C_{13} (as well as the glycosidic bonds between the sugar moieties, which are also acetals) and the ester linkage at C_{19} may be subjected to hydrolysis at low pH. Clos *et al.* found no indication of decomposition of RebA and Ste after a week of exposure to sunlight at temperatures around room temperature. These results (Clos *et al.*, 2008) contradict those of an earlier publication (Chang & Cook, 1983). The reason for this discrepancy might be an analysis protocol that was more prone to error than the one that was used by Clos *et al.* (2008)

Solid stevioside is stable up to a temperature of 140 °C, for a period of one hour. In aqueous solution, this sweetener is stable in the pH interval between 2 and 10 at a temperature of 80 °C and for a period of up to 4 h (Kroyer, 1999; Kroyer, 2010). Kroyer also investigated the effect of several acids on the stability of stevioside. Phosphoric acid has the most pronounced effect: at a concentration of 1 % phosphoric acid, a loss of 30 % is noted after 120 days.

Wölwer-Rieck *et al.* (2010) compared the stability of Ste and RebA for use in soft-drinks. According to their results, RebA is more stable than Ste. Degradation of up to 70 % was observed after 72 h of storage at 80 °C.

In a more general study, the feasibility of high purity RebA for use as a sweetener was investigated (Prakash *et al.*, 2008). In line with the results by Wölwer-Rieck, Prakash *et al.* found RebA to be stable at pH greater than 2. They found no evidence of decomposition when RebA was added to yoghurt or white cake.

So, it appears that the present study is the first more or less extensive report on the stability of steviol glycosides added to different food categories.

Methods and materials

SVGly addition

A measured amount of up to four different formulations of steviol glycosides was added to each of the food categories. These additions are indicated as “RebA” (nearly pure RebA) ; “Ste” (high concentration of Ste) ; “SV” (a commercial mixture of SVGly) and the “mixture” formulation (a mixture of 80 % “RebA” and 20 % “Ste”). The composition of each of these formulations is given in Table 2. All processed samples were analyzed within five working days after processing.

Concurrent with the chemical analysis, a trained panel evaluated sweetness and overall taste. The results of this work will be published elsewhere.

Table 2: Concentrations of the added SVGly formulations (Amery *et al.*, 2010)

	Concentration of … /(ppm)									
Formulation	**RebA**	**Ste**	**RebF**	**RebC**	**DulA**	**Peak 6(*)**	**Rub**	**RebB**	**SteB**	**TOTAL**
"RebA" formulation	96.29	0.09	0.31	0.25	0	0	0	0.29	0	97.23
"Ste" formulation	6.33	83.54	0.57	0.47	0	0	0.55	0.63	0	92.09
"SV" formulation	32.55	49.81	1.23	7.31	2.06	0.76	1.1	0.76	0.91	96.49

(*) Peak 6: a steviol glycoside, presumably RebG (Amery *et al.*, 2010)

Food processing

Samples of semi-skimmed milk, soy drink, fermented milk drink and ice cream were processed in the pilot plant of ILVO, as published elsewhere (Amery *et al.*, 2010). Drinks were stored at 6 °C and 20 °C for 20 weeks. Ice cream was stored at -18 °C for 12 weeks.

Full-fat and skimmed set yoghurt were also processed in the pilot plant. Sugar in the samples was partly replaced by one of the two mixtures: the "RebA"-formulation and the "SV"-formulation (see Table 2 for the definition; Amery *et al.*, 2010). The yoghurt was packaged immediately after inoculation with the starter culture and incubated at 43 °C until pH 4.65 was reached. The samples were stored at 6 °C for 35 days.

Jam was prepared from frozen strawberries, maltitol, steviol glycosides ("RebA" or "SV"-formulation; Table 2) according to Amery *et al.* (2010). Pectin and potassium sorbate were then added to the mixture. The strawberry jam was heated for 15 min until a Brix value of 63 – 65 ° was obtained. The jam was stored in glass jars for 6 months at room temperature in the dark and in light, and at 4 °C.

Dry biscuits containing margarine, $NaHCO_3$, flour, distilled water, polyols (isomalt or maltitol), Fibersol-2 and "RebA" (according to Amery *et al.*, 2010) were prepared at University College Ghent (Faculty of Biosciences and Landscape Architecture). The biscuits were baked at 185 °C for 14 min and stored for 4 weeks at room temperature.

Sample preparation

We refer to our previous publication (Amery *et al.*, 2010) for the details of the sample preparation of the semi-skimmed milk, soy drink, ice cream, fermented milk drink, full-fat and skimmed set yoghurt. In short, the fat was removed by centrifugation and the proteins were precipitated by addition of acetonitrile (ACN),

and removed by centrifugation. The SVGly were then concentrated by solid phase extraction, SPE, (Hypersep C_{18}, 500 mg/3 mL from Thermo Scientific, Waltman, USA) on C_{18} cartridges.

A sample of about 1 g of dry biscuits was ground in a mortar and extracted with about 20 mL of 95 % ethanol at 40 °C for 30 min. The mixture was filtered over a fluted filter-paper and the ethanol evaporated on a rotary evaporator. The residue was dissolved in 50 mL of 10 % ACN and centrifuged for 12 min at 15000 rpm on a Biofuge Strator from Heraeus Instruments in order to remove the fats and proteins. The supernatant was filtered over a filter-paper and concentrated on SPE. The cartridges were conditioned with 5 mL MeOH (Acros, Beerse, Belgium), and rinsed with 10 mL ultra pure water, made "in-house" using a Simplicity instrument from Millipore (Billerica, USA). After loading the sample, the column was rinsed with 10 mL UP water and with 5 mL 20 % ACN. The steviol glycosides were finally eluted with 5 mL 60 % ACN.

Samples of about 20 g of jam were weighted in a 50 mL falcon tube. A volume of 30 mL of water was added, and the mixture was well vortexed and placed in an ultrasonic bath for 15 min. After centrifugation (8000 g, 15 min) the supernatant was transferred to a measuring cylinder. The pellet was extracted a second time with 30 mL of water and a third time with 20 mL of water. The volume of the combined supernatants was recorded. A sample of 1 mL was transferred to a microcentrifuge tube and centrifuged (10000 g; 5 min); 250 µL hereof were used for HPLC analysis. All analyses were done in triplicate.

Analysis

All samples were analyzed with a HPLC apparatus (Thermo Scientific, Waltman, USA) consisting of an SCM1000 vacuum degasser, a P4000 pump, an AS1000 auto-sampler with a fixed injection volume of 20 µl and an UV6000LP diode array detector with a flow-cell of 10 µL and a path-length of 5 cm. Separations were done on two ODS Hypersil Columns (each 20 x 0.3 cm; 5 µm) placed in series. All samples were eluted by a linear gradient using 25 mM H_3PO_4 (solvent A) and ACN (solvent B) as eluent, as follows: 0 min: 30 % B ; 10 min: 40 % B ; 20 min: 80 % B; 30 min, 80 % B. UV Spectra were recorded between 195 and 360 nm for identification purposes, and the compounds were quantified at 200 nm.

Quantification

Steviol glycosides were quantified with an external calibration graph based on RebA as the only standard, as described elsewhere (Amery *et al.*, 2010). The analytical procedure was checked using standard addition. As an example, the standard addition curve of full-fat set yoghurt is shown in Figure 1.

The standard addition yielded a concentration of 47.85 ppm; the external calibration gave 46.84 ppm, a difference of 1.43 %. As a consequence, we can be assured that our analysis gives us accurate and precise results.

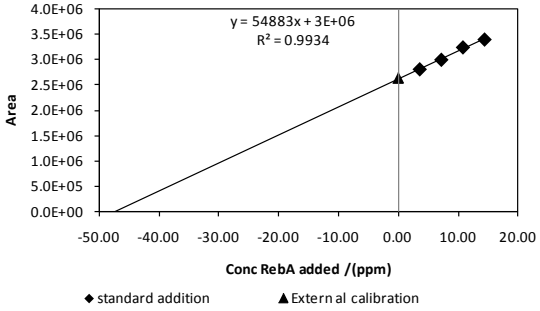


Figure 1: Standard addition curve of full-fat set yoghurt

Results and discussion

Recovery

The recovery was tested for every category of food, by spiking a sample with a known amount of RebA. The different concentrations of RebA reflect the concentrations of SvGly that are needed to give a sufficiently sweet taste to the foodstuff. As can be seen in Figure 2, the recovery, based on the concentration of RebA, ranges between 95 and 103 %.

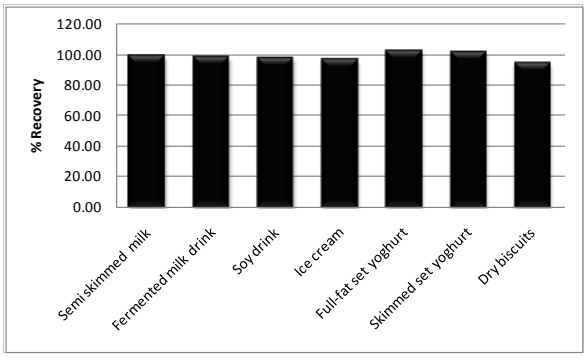


Figure 2: Recovery of RebA from the different food samples

Stability of SVGly in semi-skimmed milk

Samples of semi-skimmed milk were sweetened with all four SVGly formulations (see Table 2). The samples were stored at 6 °C and 20 °C for 20 weeks. The results are shown in Figure 3.

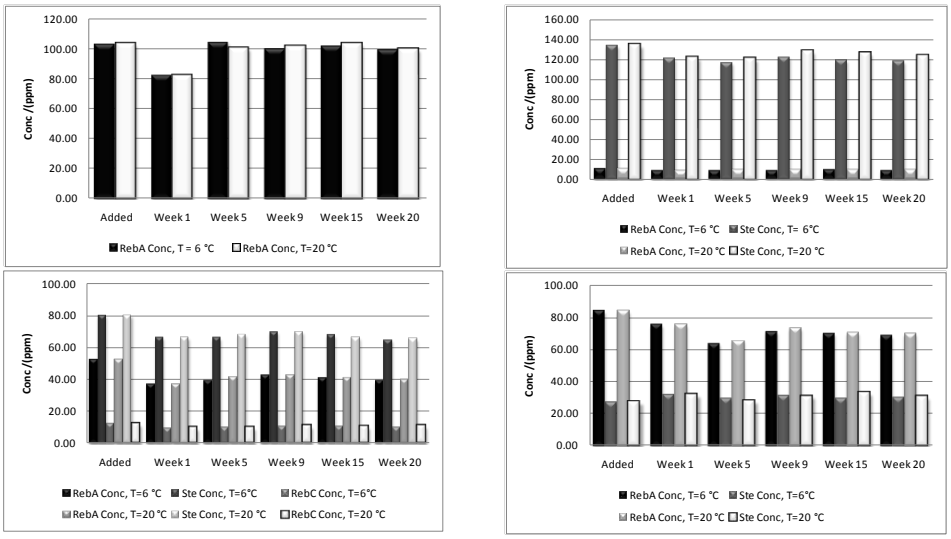


Figure 3: Stability of SVGly in semi-skimmed milk. Top left: "RebA"-formulations, top right: "Ste"-formulation, bottom left: "SV"-formulation, bottom right: "mixture"-formulation

As is evident from Figure 3, the steviol glycosides remain perfectly stable at both temperatures for 20 weeks.

Fermented milk drink

The results for the samples of fermented milk drink are depicted in Figure 4.

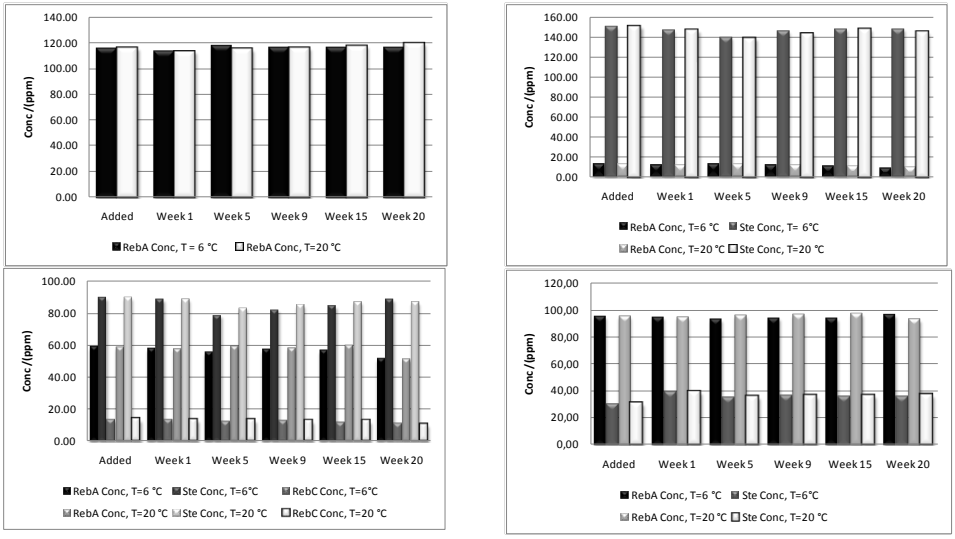


Figure 4: Stability of SVGly in fermented milk drink. Top left: "RebA"-formulations, top right: "Ste"-formulation, bottom left: "SV"-formulation, bottom right: "mixture"-formulation.

The samples of fermented milk drink were formulated and stored in an analogous manner as the semi-skimmed milk. There is no sign of decomposition in these samples.

Soy drink
Samples of soy drink were also formulated and stored as the previous samples.

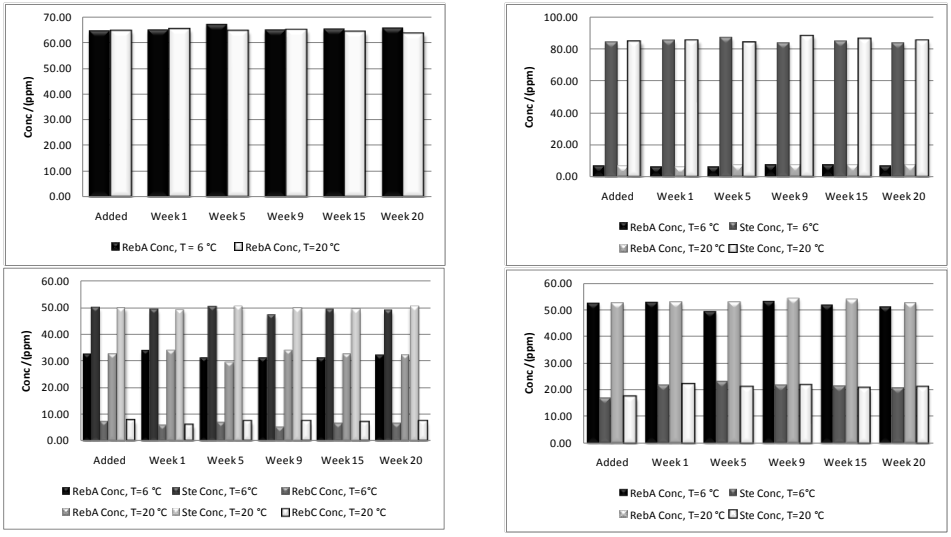


Figure 5: Stability of SVGly in soy drink. Top left: "RebA"-formulations, top right: "Ste"-formulation, bottom left: "SV"-formulation, bottom right: "mixture"-formulation.

Here, again, there is no decomposition of the added steviol glycosides within this time-frame.

Ice cream
Samples of ice cream were sweetened with all four formulations (see Table 2). The ice cream was made with either maltitol or erytritol as bulk addition. In Figure 6, only the results with maltitol are given, but those with erytritol were analogous. There is no degradation of the steviol glycosides, which is, of course, in the line of expectation for samples that were stored in the dark at such a low temperature.

Set yoghurt
Only the "RebA" and "SV" formulations were added to the full-fat and skimmed set yoghurt. Although these are acidic foodstuffs, the pH does not drop low enough to cause the steviol glycosides to hydrolyse (Kroyer, 1999; Kroyer, 2010; Prakash *et al.*, 2008). Concurrent with the chemical analysis, the pH was also checked for each sample. The pH remained constant during the entire sampling period (results not shown).

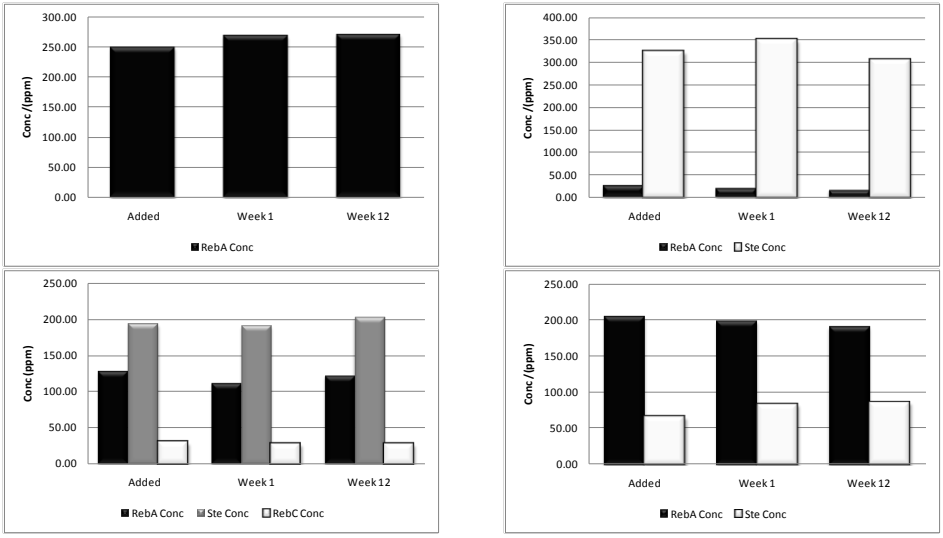


Figure 6: Stability of SVGly in ice cream. Top left: "RebA"-formulations, top right: "Ste"-formulation, bottom left: "SV"-formulation, bottom right: "mixture"-formulation

Figure 7 and Figure 8 indicate that the steviol glycosides remain perfectly stable over the period investigated. Evidently, there is no increase in the concentrations of steviol or of other, smaller, steviol glycosides (results not shown).

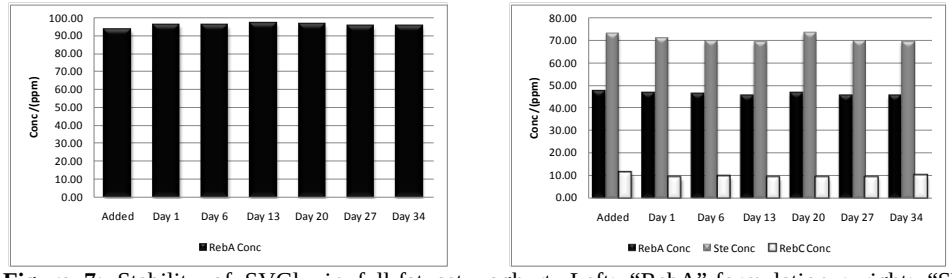


Figure 7: Stability of SVGly in full-fat set yoghurt. Left: "RebA"-formulation ; right: "SV"-formulation

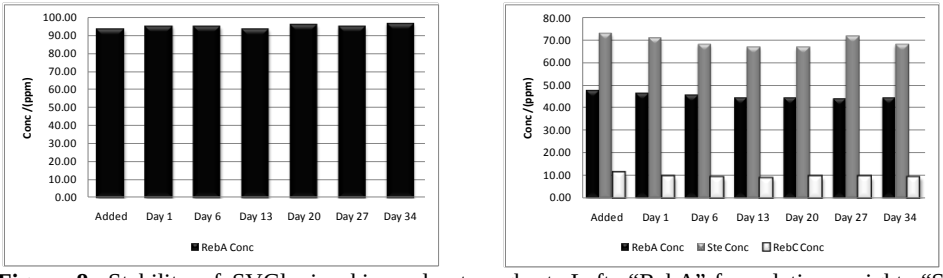


Figure 8: Stability of SVGly in skimmed set yoghurt. Left: "RebA"-formulation ; right: "SV"-formulation.

Dry biscuits

Initially, there were some reservations about the stability of the steviol glycosides in dry biscuits, because they were baked at 185 °C, and the reported upper temperature limit is 140 °C (Kroyer, 1999; Kroyer, 2010). Therefore, only one formulation, "RebA", was added. According to the literature, this is the more stable of the prominent steviol glycosides (Woelwer-Rieck *et al.*, 2010).

Apparently, the actual temperature inside the biscuits does not reach the set oven temperature, because, according to Figure 9, there is no sign of decomposition of the RebA.

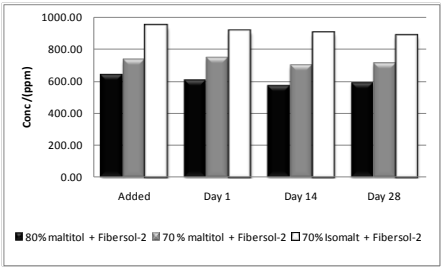


Figure 9: Stability of SVGly in dry biscuits "RebA"-formulation

Jam

Two formulations ("SV" and "RebA") and three storage conditions (room temperature in the dark and in ambient light, and 4 °C) were applied for the jam samples. As is evidenced from Figure 10, there is no breakdown of the steviol glycosides under these conditions.

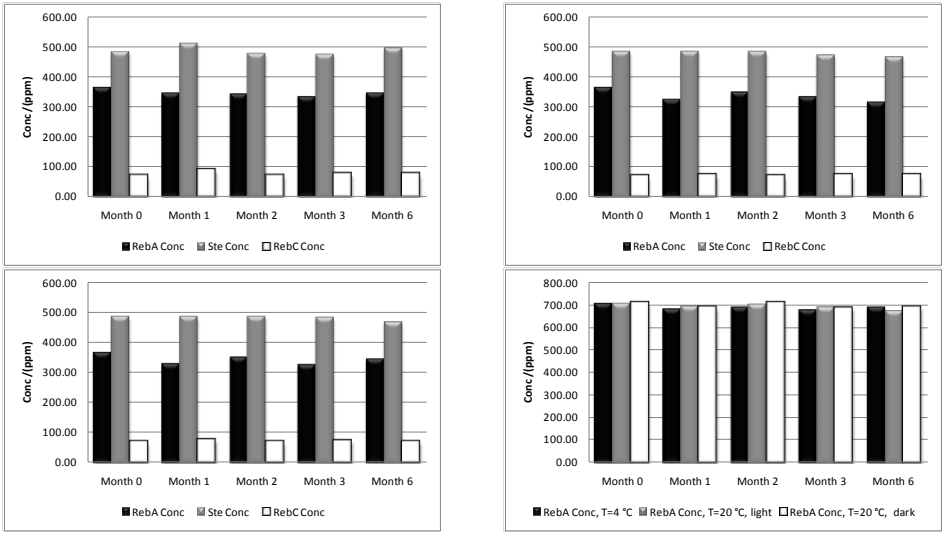


Figure 10: Stability of SVGly in jam. Top left, top right, bottom left: "SV"-formulation resp. at 4 °C; room temperature in the light and room temperature in the dark. Bottom right: "RebA"-formulation.

Conclusions

The stability of several steviol glycosides was tested in a diverse range of food categories. No sign of decomposition was found under any of the investigated circumstances. So, one can be assured that the addition of steviol glycosides to food will not alter the quality or the normal shelf life of the food.

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